Regulating the regulator: negative regulation of Cbl ubiquitin ligases

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Cbl proteins are regulators of signal transduction through many pathways and, consequently, regulate cell function and development. They are ubiquitin ligases that ubiquitinate and target many signaling molecules for degradation. The Cbl proteins themselves are regulated by an increasingly complex network of interactions that fine-tune the effects that Cbl proteins have on signaling. The negative regulation of Cbl protein function can occur via cis-acting structural elements that prevent inappropriate ubiquitin ligase activity, degradation of the Cbl proteins, inhibition without degradation owing to interaction with other signaling proteins, deubiquitination of Cbl substrates, and regulation of assembly of the endosomal ESCRT-I complex. Defects in the regulatory mechanisms that control Cbl function are implicated in the development of immunological and malignant diseases.

Introduction

Ubiquitination of proteins has a fundamental role in regulating diverse cell processes and occurs via the sequential activation and conjugation of ubiquitin to target proteins by the ubiquitin activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin protein ligase (E3) [1]. The E3 enzyme confers specificity to the ubiquitination process and directs the conjugation of ubiquitin to one or more lysines on the specific target protein.

Cbl proteins are a highly conserved family of E3s that regulate signaling by tyrosine kinases in many pathways [2,3] (Box 1). The function of Cbl proteins as regulators of signal transduction pathways, largely based on their E3 activity, is well established and has been reviewed previously [2–4]. The regulation of E3 activity is crucial to cell function and development. Regulation of ubiquitination at the level of substrate recognition, particularly by substrate phosphorylation, is well known. It is becoming evident, however, that cells have evolved intricate ways to control substrate-specific ubiquitination by modulating the amounts, activities and subcellular localizations of the E3 ligases themselves.

The Cbl proteins exemplify the diverse means of negative regulation that can be used to modulate E3 activity, including cis-acting structural elements that prevent inappropriate E3 activity until the Cbl proteins interact with a substrate [5,6], degradation of the Cbl proteins [7–13], inhibition of Cbl protein function mediated by protein interactions [14–19], deubiquitination of the Cbl substrates [20,21], and negative regulation of trafficking of the ubiquitinated Cbl substrates [22]. Disruption of the normal regulatory mechanisms that control Cbl E3 function can lead to pathological conditions such as immunological and malignant diseases [16,23–26]. Thus, proper control of Cbl E3 function is essential for maintaining normal homeostasis.

Here, we review the mechanisms that negatively regulate Cbl function and discuss the physiological implications of their failure. Although we focus on the Cbl proteins, it is likely that many E3s are regulated by similar mechanisms.

Structural regulation

In cells, Cbl proteins seem to be inactive E3s until encountering the activated kinase that they will degrade. On their interaction with the active kinase, the E3 activity of all of the mammalian Cbl proteins is induced, leading to ubiquitination and downregulation of the kinase [27–29]. Although the mechanism of this activation has not been determined precisely, the interaction between the Cbl protein and its target is crucial to induction of the Cbl E3 activity [27,29]. Investigation of the induction of c-Cbl E3 activity by Fyn kinase has shown that several Src homology domain 2 (SH2) and domain 3 (SH3) interactions are necessary for E3 activation [5].

In vitro studies of c-Cbl and Cbl-b suggest that phosphorylation of a tyrosine in the linker region between the tyrosine-kinase-binding (TKB) and RING finger domains alters the conformation of the Cbl protein, leading to E3 activation [6,27] (Figure 1). X-ray crystallography analysis of the c-Cbl protein shows, however, that the linker region tyrosine is buried within the molecule and has a structural role [30]. In this model, the linker region tyrosine would be unavailable for phosphorylation without a conformational change in the protein. It is possible that the interaction between Cbl and its substrate results in a structural rearrangement that exposes the linker tyrosine and enables its phosphorylation. Deletion of the tyrosines in the linker region abrogate c-Cbl E3 activity and render the c-Cbl protein oncogenic, further
Box 1. The Cbl proteins

The Cbl proteins are present in metazoans including worms, insects and vertebrates [98] (Figure I a). There are three mammalian Cbl proteins, c-Cbl, Cbl-b and Cbl-c (also known as Cbl-3), encoded by separate genes. Here, we use the term ‘Cbl protein’ to refer to the Cbl proteins as a group. When referring to one of the three mammalian Cbl proteins, we use the specific name listed above.

Phylogenetic analysis reveals several clusters of Cbl proteins. The first cluster consists of the roundworm Cbl proteins. The mammalian Cbl-c proteins cluster together and are most closely related to the other short Cbl proteins, the roundworm Cbl proteins. The next cluster contains the fruityfly and mosquito Cbl proteins. The Cbl protein from sea squirt (the most primitive chordate) lies between the short Cbl proteins and the higher chordates. The final two clusters contain the c-Cbl proteins and the Cbl-b proteins, respectively, from fish, chicken, rodent and human. The c-Cbl and Cbl-b proteins are equally related to the sea squirt and insect proteins by this analysis. On the basis of a comparison of the complexity and genomic organization of the Cbl gene family and the predicted Cbl proteins from various species, it can be concluded that the three mammalian Cbl genes arose by two duplications of an ancestral gene.

Previous database searches have not identified Cbl genes in single-cell organisms or in plants; however, a putative Cbl-like gene has been recently described in the genome of the social amoeba Dictyostelium discoideum, suggesting that Cbl-like genes might have originated in unicellular eukaryotes [99]. All Cbl proteins contain a highly conserved N-terminal tyrosine-kinase-binding (TKB) domain and a C3HC4 RING finger domain (Figure I b). The TKB domain mediates interactions between Cbl proteins and phosphorylated tyrosines on other proteins. The RING finger is the catalytic domain responsible for E3 activity. Cbl proteins, except v-Cbl and d-CblS, have proline-rich domains that mediate interactions with SH3-containing proteins. c-Cbl and Cbl-b share additional areas of homology in the C-terminal half of the proteins, including more extensive proline-rich regions, regions that become heavily tyrosine-phosphorylated, and an ubiquitin-associated domain.

Figure I. The Cbl proteins. (a) Phylogenetic analysis was done with the ClustalW 1.81 program using Quartet Puzzling and Gene Studio [100]. Results are shown as a rectangular cladogram drawn with TreeView [101]. Reprinted, with permission, from Ref. [98]. (b) Domain structure of the Cbl proteins. v-Cbl, the GAG–Cbl fusion protein of the Cas NS-1 murine retrovirus; c-Cbl, the human proto-oncogene of v-Cbl; Cbl-b, the second human Cbl protein; Cbl-c, the third human Cbl protein; d-CblL and d-CblS, the long and short spliced isoforms, respectively of D. melanogaster Cbl protein; Sli-1, the C. elegans Cbl protein. The tyrosine-kinase-binding (TKB) domain comprises a four-helix bundle (4H), an EF hand (EF) and an SH2 domain (SH2). The linker (L), RING finger (RF), proline-rich (P) and ubiquitin-associated (UBA) domains are indicated. Regions in the C-terminal of c-Cbl and Cbl-b that are phosphorylated on tyrosines are indicated (pY).
confirming the importance of this region in regulating Cbl protein function [31].

**Regulation by degradation**

Many E3 proteins, such as MDM2 or SIAH1, ubiquitinate themselves and are targeted for proteasomal degradation [32,33]. Several studies have demonstrated that degradation of the Cbl proteins is a regulated process. There seem to be several distinct mechanisms, however, including auto-ubiquitination and trans-ubiquitination by other E3 ligases (Figure 2).

**Coordinated degradation of Cbl proteins and their RTK substrates**

Induction of the Cbl protein E3 activity seems to depend on interaction of the Cbl protein with its substrate; however, the mechanism that terminates the E3 activity might also be dependent on this interaction. Cbl proteins are degraded along with some of their substrates, which might function to prevent the activated Cbl enzyme from indiscriminately degrading other proteins. This type of degradation was first observed for c-Cbl and Cbl-b proteins in studies of epidermal growth factor receptor (EGFR) downregulation: EGF induces a loss of Cbl protein that parallels the decrease in EGFR [7]. Similar findings have been observed for Cbl-c (S. Lipkowitz et al., unpublished). Subsequently, such a loss of Cbl protein has been described in studies of c-Cbl- and Cbl-b-mediated downregulation of the Kit receptor tyrosine kinase (RTK) [8].

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**Figure 1.** Activation of Cbl protein E3 activity. The N-terminal region of the Cbl proteins autoinhibits their E3 activity. On recruitment of the Cbl protein to an activated tyrosine kinase (TK), the tyrosine kinase phosphorylates the Cbl protein on a tyrosine in the linker region between the tyrosine-kinase-binding (TKB) domain and the RING finger (RF) domain, resulting in a conformational change and in activation of the Cbl protein E3 activity. The Cbl protein RF then catalyses the transfer of ubiquitin (Ub) from the E2 associated with the RF to a lysine residue on the TK.

**Figure 2.** Regulation by degradation. Cbl proteins are degraded by various mechanisms, including their coordinate degradation along with the EGFR, degradation by Src and degradation by Nedd4, as shown here. The Cbl protein ubiquitinates both itself and the EGFR on recruitment to the activated EGFR. The Cbl protein is degraded along with the EGFR in the lysosome. Cbl proteins are degraded by the proteasome on interaction with activated Src and this degradation is mediated, at least in part, by the Cbl E3 activity. Nedd4 and other HECT E3 enzymes target Cbl proteins for proteasomal degradation. This pathway is dependent on the HECT E3 activity and is independent of the Cbl protein E3 activity.
For EGF-induced Cbl protein degradation, the Cbl protein must bind to the activated EGFR and its E3 function must be intact [7]. The data are most consistent with ubiquitin-mediated degradation of the Cbl molecules that are directly interacting with the EGFR, suggesting that the E3 activity of Cbl proteins is shut off by degradation of the activated Cbl protein along with the EGFR.

This coordinated degradation of Cbl proteins has not been observed universally with other Cbl RTK substrates. The detection of EGF-induced Cbl degradation is, however, dependent on the ratio of Cbl protein to the EGFR [7]. Thus, at high levels of Cbl proteins relative to the substrate, degradation of the Cbl protein might be missed because only a small proportion of the Cbl protein would interact with the substrate and subsequently be degraded. Alternatively, degradation of the Cbl protein along with the substrate might be substrate-specific. For example, c-Cbl has been shown to be ubiquitinated on interaction with the colony-stimulating factor (CSF) receptor, but this ubiquitination is reversible and does not result in degradation of c-Cbl [34,35].

**Degradation by Src kinases**

Activated forms of Src induce ubiquitination and degradation of c-Cbl [10,29], which in turn prevent downregulation of the activated EGFR by c-Cbl [10]. Src-induced degradation has not been demonstrated for Cbl-b or Cbl-c, however, all of the Cbl proteins can induce downregulation of Src family kinases [5,9,28,29]. Work with Src suggests that the interaction between Src and c-Cbl induces ubiquitination of both proteins [2,29]. Studies with the Src family kinase Hck have shown degradation of both c-Cbl and Hck on interaction of the proteins [36].

C-Cbl colocalizes with activated Src in tubulo-vesicular structures similar to those seen for the activated EGFR and c-Cbl, suggesting that Src induces degradation of c-Cbl by the endocytic pathway [10]. These data indicate that, like the coordinated degradation described for Cbl proteins and the EGFR, the interaction of Cbl proteins with Src kinases induces ubiquitination and degradation of both proteins. In addition, degradation of c-Cbl by Src might be mediated by other E3s because c-Cbl mutants that lack E3 activity are still degraded by coexpression with activated Src [10].

In experimental systems, activated Src potentiates the transforming activity of the EGFR, and this potentiation is due, at least in part, to degradation of the Cbl proteins by activated Src [10,37,38]. Src kinase activity is higher in many different human epithelial malignancies than in normal tissues (reviewed in Ref. [39]). An increase in Src activity would be predicted to cause Cbl degradation, which might contribute to the development or progression of these cancers.

**Degradation of Cbl proteins by HECT E3 ligases**

The yeast HECT E3, Rsp5p, and its mammalian orthologs, Nedd4 and Nedd4-2, ubiquitinate various membrane proteins, including transporters and the insulin-like growth factor-1 (IGF-1) receptor, and target them for lysosomal degradation [40,41]. Nedd4 is a member of a family of E3s that are characterized by a HECT E3 catalytic domain and a variable number of WW domains [42]. Colocalization of Nedd4 and c-Cbl in membrane rafts on activation of the immunoglobulin-e (IgE) receptor and biochemical interaction of the HECT E3 Itch (also known as AIP4) and Cbl-c suggest that Cbl proteins and this family of HECT E3s might interact functionally in regulating protein degradation [43,44].

Nedd4 and Itch bind all of the mammalian Cbl proteins and target them for proteasomal degradation [9]. This degradation has been found to depend on the E3 activity of the HECT E3s but not on that of the Cbl proteins. Moreover, no ubiquitination or degradation of the HECT E3 by the Cbl protein has been observed. Consistent with these findings, in cells expressing the EGFR, Nedd4 prevents Cbl-mediated ubiquitination and downregulation of the EGFR, which in turn leads to persistent downstream signaling by this receptor. These findings establish that the Cbl RING finger E3s can be substrates not only for auto-ubiquitination but also for ubiquitination by HECT E3s. They also suggest the potential for an additional level of regulation of Cbl protein function.

**CD28-induced Cbl-b degradation**

Studies of Cbl-b-deficient mice have shown that Cbl-b sets the stimulation threshold of T lymphocytes by negatively regulating the CD28 costimulation pathway and thereby controlling the induction of anergy [45,46]. Activation of the T-cell antigen receptor (TCR) in the absence of CD28 costimulation results in upregulation of Cbl-b mRNA and protein, which in turn leads to inhibition of cytokine production, inhibition of proliferation, and subsequent induction of anergy [45,46]. Homozygous deletion of Cbl-b results in an increase in proliferation in the absence of CD28 costimulation and the development of autoimmunity [45].

In normal T-lymphocytes, stimulation of CD28 results in ubiquitination and degradation of Cbl-b in a dose-dependent fashion that correlates with the degree of proliferation induced [11,45,47]. By contrast, c-Cbl is neither ubiquitinated nor degraded on CD28 stimulation [11]. The mechanism that accounts for the selective degradation of Cbl-b on activation of CD28 is not known. It is not clear whether Cbl-b is degraded in an autocatalytic fashion or whether it is the substrate for degradation by another E3 activity (e.g. the E3s Itch and GRAIL are induced by T-cell stimulation [45]). Notably, CD28-mediated activation of T lymphocytes from older rats does not stimulate downregulation of Cbl-b [47]. Thus, the decline in T-lymphocyte responsiveness that occurs with aging might be related to loss of negative regulation of Cbl-b by CD28 costimulation. The basis for the loss of Cbl-b degradation with age is not known.

**Interaction with Sts-1 and Sts-2**

Sts-1 and Sts-2 (also known as TULA) are recently identified c-Cbl-interacting proteins that can regulate the effects of Cbl on RTK signaling [12,13]. These proteins contain an N-terminal ubiquitin-associated (UBA) domain that interacts with ubiquitin, an SH3 domain that...
interacts constitutively with the proline-rich regions of c-Cbl, and a phosphoglycerate mutase domain that mediates homodimerization. On activation of the EGFR, Sts-1 and Sts-2 are recruited to the EGFR and inhibit c-Cbl-mediated ubiquitination and downregulation of this receptor. Similar inhibition of platelet-derived growth factor receptor (PDGFR) downregulation has been described for Sts-2 [13]. So far, no studies have described interactions between Sts proteins and either Cbl-b or Cbl-c.

Coexpression of Sts-2 and c-Cbl results in ubiquitination and degradation of c-Cbl [12]. Degradation of c-Cbl by Sts-2 is increased by activation of the EGFR. The Sts-2-mediated loss of c-Cbl protein requires intact c-Cbl TKB and RING finger domains. These data are consistent with auto-ubiquitination and degradation of the c-Cbl proteins that are interacting with the activated kinase. How the Sts proteins selectively cause degradation of c-Cbl while sparing the EGFR is not clear. The UBA domain of Sts-2 is required for inhibiting c-Cbl-mediated downregulation of the EGFR and for degradation of c-Cbl. The role of the UBA domain of Sts-2 is unclear. It is possible that it binds to and masks the ubiquitinated EGFR and prevents trafficking of the EGFR to the endocytic pathway. Consistent with this, overexpression of an isolated UBA domain interferes with ubiquitin-mediated processes including EGFR downregulation [48].

The physiological role of the Sts proteins is not clear. Sts-1 is expressed ubiquitously and could regulate signaling in both epithelial and hematopoietic cells [49]. Overexpression of Sts-2 inhibits c-Cbl-mediated downregulation of the EGFR and the PDGFR. However, Sts-2 is expressed predominantly in hematopoietic cells and thus would not regulate signaling by these RTKs [12,13,49]. Overexpression of Sts-2 enhances TCR signaling in Jurkat T cells, and reducing Sts-2 by short interfering RNA (siRNA) results in attenuated TCR signaling [12]. c-Cbl inhibits TCR signaling in Jurkat T-cells, and overexpression of Sts-2 reverses this c-Cbl-mediated inhibition. Contrary to these results, mice lacking both Sts proteins are hyperresponsive to TCR stimulation, resulting in an increase in both cytokine production and susceptibility to autoimmunity [49]. The discrepancy between the biochemical studies based on Sts-2 in Jurkat T-cells, which suggest that these proteins are positive regulators of signaling by inhibiting Cbl proteins, and the studies in mice, which show that the loss of both Sts proteins leads to hyperactive TCR responses, remains to be reconciled.

**Regulation without degradation**

As we describe here, several proteins can effectively inhibit downregulation of the activated EGFR by Cbl proteins without obviously affecting the quantities of Cbl proteins (Figure 3).

**Sprouty**

The first protein found to compete with the EGFR for Cbl protein binding was Sprouty2. Originally Sprouty was the first protein found to compete with the EGFR for Cbl and mitogen-activated protein kinase (MAPK) signaling in Drosophila melanogaster [15].

Similarly, the mammalian orthologs of Sprouty inhibit MAPK activation induced by the fibroblast growth factor receptor [15]. By contrast, human Sprouty1 and Sprouty2 have been shown to enhance EGFR-mediated activation of MAPK [15].

c-Cbl and Cbl-b bind constitutively to Sprouty via the RING finger domain in a weak interaction [15,50]. On activation of the EGFR, Sprouty2 becomes phosphorylated on Tyr55 and c-Cbl binds to the phosphorylated Sprouty protein via its SH3 domain. The formation of this trimeric Cdc42- p85Cool-1-Cbl complex prevents interaction of the Cbl protein with the EGFR. On hydrolysis of GTP, Cdc42 dissociates from p85Cool-1 and the Cbl protein is released and can associate with and ubiquitinate the EGFR. Other proteins (such as cortactin, HPV16 E5, Alix and SHIP2) prevent Cbl-mediated downregulation of the EGFR without degradation of the Cbl protein, but the mechanisms by which they inhibit Cbl function are not clear.
Recent data suggest that Sprouty2 (or Sprouty1) forms a trimolecular complex with c-Cbl and CIN85 that is required for inhibiting c-Cbl-mediated EGFR degradation. Sprouty4, which has a conserved c-Cbl-binding site but lacks the CIN85-binding site, does not inhibit c-Cbl function [54]. The phosphorylation-dependent interactions between Sprouty proteins and Cbl-b or Cbl-c have not been investigated, but the high homology among the Cbl TKB domains suggest that all Cbl proteins would behave similarly.

Recent studies indicate that Sprouty2 positively regulates the transformation of fibroblasts by activated H-Ras [55]. A reduction in Sprouty2 protein mediated by RNA interference results in a decrease in transformed phenotype. Studies of EGFR activation have shown that loss of Sprouty2 expression leads to a decrease in EGFR activity owing to enhanced downregulation of this receptor. In addition, a survey of human cancer cell lines has shown increased Sprouty2 expression in these cells [55]. These results are consistent with the idea that Sprouty2 functions as a positive regulator of EGFR function and suggest that overexpression of Sprouty2 could contribute to carcinogenesis by preventing Cbl-mediated downregulation of the EGFR.

Cdc42
Cdc42 is a second example of a protein that competitively inhibits downregulation of the EGFR by Cbl proteins. Cdc42 is a Ras-related GTPase that is active in various cellular processes [56]. Stimulation by EGF results in activation of Cdc42, which in turn binds to the protein p85Cool-1 (also known as β-Pix) [16]. The SH3 domain of p85Cool-1 interacts with the proline-rich domain of c-Cbl or Cbl-b [16]. Thus, EGFR activation results in a complex of Cdc42, p85Cool-1 and Cbl protein. This complex sequesters the Cbl protein and prevents it from binding to the activated EGFR, resulting in decreased ubiquitination and downregulation of the activated EGFR and prolonged downstream signaling by the EGFR.

The interaction with Cdc42 could provide a temporal switch for Cbl proteins. On EGF stimulation, Cdc42 would become activated and sequester Cbl proteins, enabling EGFR signaling to proceed. Conversion of Cdc42 to the inactive state by GTP hydrolysis would result in dissociation of the Cdc42–p85Cool-1–Cbl complex, enabling Cbl proteins to bind to the EGFR and mediate its downregulation.

Aberrant activity of Cdc42 might contribute to the overexpression of the EGFR seen in many human cancers. Constitutively active, transforming mutants of Cdc42 sequester Cbl, resulting in enhanced mitogenic signaling by the EGFR [16]. Mutations that disrupt the Cdc42–p85Cool-1–Cbl complex prevent transformation by activated Cdc42. Thus, the sequestration of Cbl proteins by activated Cdc42 seems to be essential for transformation by activated Cdc42. Notably, Cdc42 and p85Cool-1 are overexpressed in some human cancers [57–61].

Cortactin
Recently, overexpression of cortactin has been shown to prevent downregulation of the EGFR [17]. Cortactin is a multidomain protein that associates with the actin-related protein Arp2/3 and regulates actin polymerization [62]. Overexpression of cortactin decreases phosphorylation of c-Cbl and its association with the activated EGFR, in parallel with a reduction in ubiquitination and degradation of this receptor. The mechanism by which this occurs is unclear.

In model systems, overexpression of cortactin increases cell motility and metastasis [62]. In human breast cancer and squamous carcinoma of the head and neck, the cortactin protein is frequently overexpressed owing to gene amplification [62]. This amplification is associated with a poor prognosis [62]. The precise role of cortactin in cancer progression is not defined, but it is possible that its inhibition of Cbl-mediated downregulation of RTK signaling is at least partially responsible.

Alix
The protein Alix (also known as AIP1) can interact with both CIN85 and endophilins [63–65]. Because CIN85 and endophilins associate with Cbl proteins and have been shown to mediate endocytosis of activated RTKs, the role of Alix in EGFR downregulation has been investigated [18]. Alix inhibits c-Cbl- and Cbl-b-mediated ubiquitination and downregulation of the EGFR by an unknown mechanism. There is evidence of a decrease in EGFR-induced phosphorylation of Cbl proteins when Alix is overexpressed [18]. In addition, there is less phosphorylation of EGFR Tyr1045, the binding site for the Cbl TKB domain, and a decrease in the amount of Cbl protein associated with the EGFR on activation [18].

Alix associates with the EGFR independent of activation and can enhance the binding of CIN85 and endophilins to the activated EGFR; however, the association between Cbl proteins and CIN85 decreases in the presence of excess Alix. These data suggest that the Alix protein complexes prevent recruitment of the Cbl proteins to the activated EGFR and also sequester CIN85 and endophilins away from Cbl proteins, thereby preventing Cbl-mediated ubiquitination and downregulation of the EGFR.

HPV16 E5
Human papillomavirus type 16 (HPV16) is highly associated with human cervical cancer [66]. Overexpression of the EGFR in HPV16-induced cervical cancer is an indicator of a poor prognosis [67]. The HPV16 E5 protein inhibits lysosomal degradation of the activated EGFR and increases EGFR recycling to the cell surface [68].

Recent data show that the E5 protein prevents recruitment of c-Cbl to the activated EGFR and prevents EGFR ubiquitination, resulting in prolonged signaling by this receptor [19]. The underlying mechanism remains unclear. The E5 protein does not seem to affect steady-state levels of c-Cbl, translocation of c-Cbl to the membrane on EGFR activation, or phosphorylation of c-Cbl [19]. The E5 protein enhances transformation of cells by the EGFR by preventing Cbl-mediated downregulation of the EGFR [19,68,69].
SHIP2
Phosphorylated phosphatidylinositol-4-phosphate 5'-kinase II (SHIP2) is a lipid phosphatase that dephosphorylates phosphatidylinositol 3,4,5-trisphosphate at the 5' position of the inositol ring to generate phosphatidylinositol (3,4)-bisphosphate [71].

Recent work has shown that ligand-induced internalization and degradation of the EGFR in HeLa cells are increased when SHIP2 expression is reduced by siRNA [72]. A reduction in SHIP2 expression results in more association of c-Cbl protein with the activated EGFR and more ubiquitination of the EGFR. SHIP2 interacts with phosphorylated c-Cbl via its SH2 domain and this interaction is increased on EGFR activation. These results suggest that SHIP2 prevents c-Cbl-mediated ubiquitination and degradation of the activated EGFR by inhibiting the recruitment of c-Cbl to this receptor. Further mechanistic details have not been described. Specifically, the role (if there is one) of phosphorylated phosphatidylinositol-4-phosphate 5'-kinase II remains to be examined.

Downstream regulators of Cbl function
Degradation of ubiquitinated RTKs requires sorting from the early endosome to the multivesicular body (MVB). This process is carried out by several ubiquitin-interacting molecules and the large multiprotein complex ESCRT-I [73]. Regulation of Cbl-mediated downregulation can occur by deubiquitination of the substrate and by regulation of ESCRT-I function.

Deubiquitination
Deubiquitinating enzymes (DUBs) remove ubiquitin from proteins and prevent ubiquitin-mediated degradation of proteins. The DUB UBPY (also known as USP8) is recruited to the activated EGFR and colocalizes with the EGFR in endosomes, where it deubiquitinates the EGFR [20] and prevents its degradation. Consistent with a role as a positive regulator of growth factor stimulation, expression of UBPY is induced in serum-stimulated cells and its loss blocks progression of the cell cycle [74]. The endosome-associated DUB AMSH can also deubiquitinate the EGFR and loss of AMSH enhances degradation of the activated EGFR [75].

Similarly, DUB-2 deubiquitinates the common cytokine receptor γc and prevents downregulation of γc by c-Cbl [21]. γc is shared by many cytokine receptors and is required for lymphocyte proliferation and survival. DUB-2 is induced by cytokine stimulation of lymphocytes [76]. Overexpression of DUB-2 in pro-B cells prolongs cytokine-induced signaling and inhibits apoptosis induced by cytokine withdrawal [77]. DUB-2 is highly expressed in T cells transformed with human T-lymphotrophic virus-1 (HTLV-1) in which cytokine signaling pathways are aberrantly active, suggesting that inhibition of ubiquitin-mediated downregulation of γc by DUB-2 contributes to transformation of T cells by HTLV-1 [77].

MVB sorting
Ubiquitinated RTKs are sorted from early endosomes into the MVB for degradation [73]. The recognition of the ubiquitinated RTKs necessitates both adaptor proteins that contain ubiquitin-binding motifs (e.g. Hrs) and the endosomal sorting complex required for transport-I (ESCRT-I) [73].

Tsg101 is a crucial component of ESCRT-I and is required to sort ubiquitinated substrates such as the EGFR to the MVB [78]. Tsg101 is itself regulated by ubiquitination: the E3 Tall monoubiquitinates Tsg101 [22]. Ubiquitinated Tsg101 does not assemble into the ESCRT-I complex, and its ubiquitinated substrates, such as the EGFR, are not sorted to the MVB. Thus, ubiquitination of Tsg101 negatively regulates Cbl-mediated downregulation of the EGFR and results in prolonged signaling by this receptor. Tsg101 was first identified as a tumor suppressor gene in mouse fibroblasts and inhibition of RTK downregulation might account for transformation caused by loss of Tsg101 [79].

Regulation of Cbl proteins and disease
Cbl proteins function in many signaling pathways and it is possible that dysregulation of Cbl protein function contributes to several pathological conditions. Evidence suggests that disruption of both Cbl protein function and the regulatory mechanisms controlling this function might contribute to immunological and malignant diseases.

Immunological diseases
Mice lacking Cbl-b develop autoimmune diseases [80,81]. Consistent with this, loss of Cbl-b increases susceptibility to autoimmune diabetes in rats [82]. Human studies of Cbl-b in individuals with type I diabetes (autoimmune diabetes) have identified a single nucleotide polymorphism in Cbl-b that might be associated with the disease [83]. The functional consequences of this polymorphism are not known.

In another autoimmune disease, systemic lupus, studies of T lymphocytes suggest that c-Cbl and Cbl-b function is attenuated and that signaling is prolonged in T cells from affected individuals [84]. The mechanism that accounts for this attenuation of Cbl function has not been characterized. Conversely, as described earlier, a failure to downregulate Cbl-b might contribute to the loss of T-cell function that occurs with aging. Aberrations in the regulatory mechanisms controlling Cbl function are likely to contribute to immunological diseases, but as yet these mechanisms have not been fully evaluated in such diseases.

Cancer
Originally, v-Cbl was identified as the transforming gene of the Cas NS-1 virus that causes B-cell lymphomas and myeloid leukemias in mice and transforms fibroblasts in culture [85,86]. Additional transforming mutations of c-Cbl have been identified both in murine lymphoma cell lines and from mutation analysis studies [87–89]. These transforming mutations all cluster in the region between the SH2 domain and the RING finger domain of c-Cbl and inactivate the E3 activity. Importantly, the Cbl proteins seem to have functional redundancy. Many in vitro studies have shown that all of the Cbl proteins can negatively
regulate tyrosine kinase function for various kinases (e.g. EGFR and Src). Mice deficient in each of the Cbl proteins are viable, but loss of both c-Cbl and Cbl-b results in embryonic lethality [90]. Thus, aberrations in the function of all of the Cbl proteins might be required to unmask transforming activity.

There are several mechanisms by which inactivation of all Cbl proteins can occur. Expression of a dominant-negative form of a Cbl protein could inactivate all endogenous Cbl proteins. The transforming variants of c-Cbl proteins are thought to work, at least in part, as dominant-negative Cbl proteins. No such transforming mutations of Cbl proteins have been found in human malignancies.

Abrogating the interaction between Cbl proteins and their target provides another means to inhibit the function of all Cbl proteins. Mutations in the Cbl-binding site of RTKs that prevent Cbl-mediated downregulation can contribute to the transforming activity of these kinases (reviewed in Ref. [91]). For example, mutations in the Cbl-binding site of the CSF-1 receptor enhance the transforming activity of this receptor [91]. Such mutations of the CSF-1 receptor have been found in children with secondary myelodysplasia and acute myeloid leukemia [92,93]. Mutations that would prevent Cbl binding to RTKs have not been described so far in human epithelial malignancies. Overexpression of ErbB-2, however, which is seen in several human epithelial malignancies, can inhibit Cbl-mediated downregulation of the EGFR by preventing Cbl binding to the heterodimers [26]. In addition, many epithelial tumors overexpress the EGFR-related peptide transforming growth factor-α (TGFα), which can function as an autocrine growth factor [94]. Unlike stimulation by EGF, TGFα stimulation results in a decrease in Cbl protein recruitment to the EGFR, a decrease in ubiquitination of the EGFR, and a decrease in degradation of the EGFR [95–97].

Inappropriate activity of the negative regulators of Cbl proteins provides another means of inhibiting the function of all Cbl proteins. As described earlier, many of the negative regulators of Cbl proteins enhance transformation by the EGFR in model systems and presumably would inhibit all Cbl proteins. Evidence indicates that several of the negative regulators of Cbl proteins (e.g. Src, Cdc42, c-Jun, and DUB-2) are aberrantly active or overexpressed in human malignancies and thus might contribute to transformation by inhibiting downregulation of RTKs such as the EGFR. Further investigation of receptor downregulation in human cancers is warranted on the basis of these observations.

Concluding remarks
The E3 activity of Cbl proteins is regulated by a complex set of interactions. Observations such as those outlined here suggest that disruption of the normal regulation of Cbl proteins might contribute to the development of immunological and malignant diseases. So far, many of the studies of Cbl proteins have relied heavily on in vitro and transfection methodologies. The challenge for the future is to understand these complex interactions under physiological conditions and to modulate these interactions as a therapeutic approach to diseases such as autoimmunity, anergy and cancer.

Acknowledgements
We thank Allan Weissman for critical review and helpful suggestions. We apologize to colleagues whose work could not be cited owing to space limitations. This work was supported by the Intramural Research Program of the National Institutes of Health, National Cancer Institute and Center for Cancer Research.

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